OCCURRENCE AND CHARACTERIZATION OF NEW POLYAMINES IN THE EXTREME THERMOPHILE *Caldariella acidophila*

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SUMMARY. Polyamines of *Caldariella acidophila* have been detected by H.V. electrophoresis and gas-liquid chromatography combined with mass spectrometry. Spermidine, thermine and caldine $NH_2CH_2CH_2CH_2NHCH_2CH_2CH_2NH_2$, a new polyamine, have been identified and their cellular concentration measured by an isotope dilution technique. The possible metabolic interrelations of such molecules are discussed.

The well known role of polyamines in stabilizing nucleic acids against thermal denaturation (1) prompted us to investigate the occurrence of such molecules in *Caldariella acidophila*, an extreme thermoacidophilic bacterium. The growth temperature (87°C) of such microorganism is remarkably high if compared to the other thermophiles reported in Literature (2-4).

Recently Tairo Oshima has reported the presence of spermine and thermine, a new tetraamine, in some thermophilic microorganisms (5); it is interesting to note in this respect that thermine appears to be present only in extreme thermophiles, whereas it is absent in moderate thermophiles or mesophiles. The role of such molecules in protein synthesis of thermophiles has also been investigated (6).

The polyamines pool of *Caldariella acidophila*, purified by ion exchange chromatography, has been analyzed by H.V. electrophoresis and by gas-liquid chromatography combined with mass spectrometry. Spermidine, thermine (1,11 diamino-4,8-diazaundecane) and 1,7 diamino-4-azaheptane, a new triamine, were detected in the cells at various stages of growth. Referring to the name of the microorganism the authors propose to attribute the trivial name of caldine to

this new natural compound. Its possible role as precursor of thermine will be discussed.

MATERIALS AND METHODS

Chemicals - Yeast extract and casaminoacid were purchased from Difco Laboratories; 1,7 diamino-4-azaheptane from Koch-Light and 1,11 diamino-4,8-diazaundecane from Eastman Kodak; putrescine, spermidine, spermine, dithiothreitol were obtained from Sigma Chemical Co.; Dowex 50W resin from Bio Rad Laboratories; [1,4-^{14}C]putrescine, [1,4-^{14}C]spermidine and [1,4-^{14}C]spermine from NEN Chemicals; trifluoroacetic anhydride (TFAA) from Pierce Chemical Co..

Microorganism - Caldariella acidophila strain MT-4 was employed. The cells were grown at 87°C pH 3.5 (7) and harvested in the late log-phase (18h) or in the stationary-phase (36h). Then were collected by continuous flow centrifugation with an Alfa Laval model LAB 102 B25 centrifuge and washed twice with 0.25M sucrose plus $5x10^{-4}$M dithiothreitol, pH 6.2.

Extraction of polyamines - The extraction of polyamines from the cells was carried out according to Inoue and Mizutani (8). The supernatant obtained after centrifugation of the perchloric acid extract of 10g wet cells was directly chromatographed on Dowex 50 (H^+ form) column (10x2cm) previously equilibrated with 0.1N HCl. Basic amino acids and other contaminants were eluted with 500 ml of 0.1M sodium phosphate buffer pH 8 containing 0.7M NaCl and then with 250 ml of 2.5N HCl. Polyamines were eluted with 125 ml of 6N HCl. The strongly acidic eluate was evaporated to dryness in rotary evaporator under reduced pressure. The residue was dissolved in 3 ml of distilled water.

Paper electrophoresis and colorimetric determination of polyamines - The electrophoresis was carried out on a Savant high voltage electrophoresis apparatus, model LT-48 A, with a distance of 45 cm between the electrodes. The separation was performed for 40 min at 3,000 volts on Whatman 1MM paper (57x46 cm) using $5x10^{-2}$M citrate buffer pH 3.4. After migration the resolved amines were visualized with ninhydrin reagent (8). The colorimetric determination was performed after elution of the spots employing standard curves (8) obtained in similar way.

Quantification of polyamines by isotope dilution - An isotope dilution technique was used because of the small amounts of polyamines present in the microorganism and for the complexity of the extraction resulting in low yields. Labeled polyamines were added directly to cell suspension before homogenization. To 10 g of wet cells were additioned: $6.75x10^5$dpm of [1,4-^{14}C] putrescine (0.12 mCi/mg), $9.85x10^5$dpm of [1,4-^{14}C]spermidine (0.035 mCi/mg) and $9.85x10^5$dpm of [1,4-^{14}C]spermine (0.03 mCi/mg). Polyamines were resolved by electrophoresis and revealed with ninhydrin. The radioactivity was measured by an Actigraph III model 1002 Nuclear Chicago and then the spots were eluted from the paper for quantitative assay (8).

Gas chromatography-Mass spectrometry of polyamines - The characterization of polyamines isolated from *Caldariella acidophila* was further performed by gas-liquid chromatography and combined gas chromatography-mass spectrometry (GC-MS). In order to obtain more volatile compounds the polyamines were converted into the correspondent trifluoroacetyl derivatives. The polyamine eluate from the cation-exchange column was completely dried and then dissolved in equal portions (1.0 ml) of CH_3CN and TFAA. The derivatization was accomplished by placing the capped sample tube in an oil bath at 100°C for 5 min. The CH_3CN

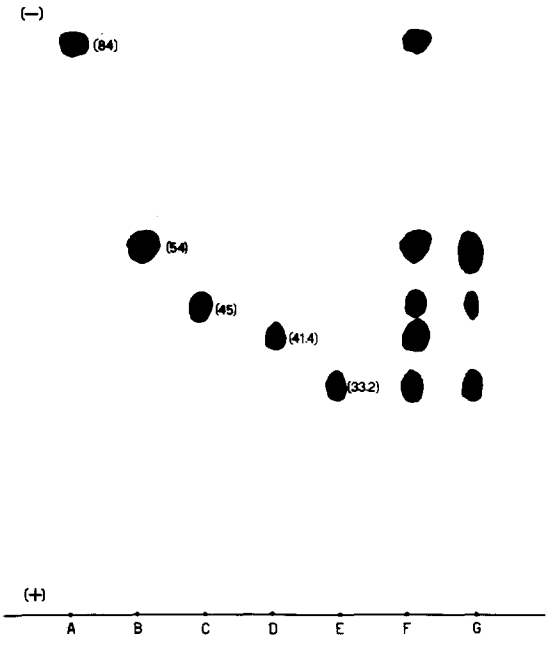


Fig.1. Paper electrophoresis of polyamine pool from *Caldariella acidophila* and reference polyamines. a) putrescine, b) spermidine, c) caldine, d) spermine, e) thermine, f) mixture of the standards, g) polyamine pool from *C. acidophila*. The numbers within brackets refer to the relative mobility expressed as cm $volt^{-1}$ h^{-1}x10^3

and TFAA were then removed by evaporation with N_2 gas at room temperature. The same technique was used for derivatization of reference compounds. A Carlo Erba Fractovap GV (standard model, with ionization detector) was employed. The Pyrex glass gas chromatographic column (150mmx3mm I.D.) was packed with 3% SE-30 on Chromosorb W silanized, 80-100 mesh. N_2 was employed as carrier gas (40 ml/min) at an operating temperature of 210°C. A MS-30 double beam AEI mass spectrometer, operating at an accelerating voltage of 4KV, with an electron energy of 3E eV, was combined with a Pye series 104 chromatograph. The gas chromatographic column was prepared as described above; He (40 ml/min) was used as carrier gas with temperature program of 8°C/min from 120° to 300°C.

RESULTS

Identification of polyamines.

H.V. Electrophoresis -The two polyamines caldine and thermine are well resolved from the correspondent analogues spermidine and spermine by paper electrophoresis with citrate buffer pH 3.4 as shown in Fig.1. Above pH 5.0 and below pH 2.5 no difference in electrophoretic mobility between caldine and spermidine, and between thermine and spermine, respectively, was detectable. The polyamines from *C. acidophila* were resolved into 3 amine-indicating spots

(Fig. 1). The two major spots corresponded to authentic spermidine and 1,11 diamino-4,8-diazaundecane (thermine), while the minor spot to authentic 1,7 diamino-4-azaheptane (caldine); spermine and putrescine are not detectable.

Gas-liquid chromatography - A complete acylation of all amino groups of polyamines was obtained in the conditions employed for derivatization. Authentic spermidine, spermine, 1,7 diamino-4-azaheptane (caldine) and 1,11 diamino-4,8-diazaundecane (thermine) were resolved by the GLC system used, with excellent peak symmetry. The mixture of polyamines obtained from *C. acidophila* was resolved into three peaks, with retention times of 1.9 min for 1,7 diamino-4-azaheptane, 2.8 min for spermidine and 12.8 min for 1,11 diamino-4,8-diazaundecane respectively. No amine with retention time of spermine (19.3 min) was observable.

Gas chromatography-Mass spectrometry - For an unambiguous identification and characterization of the polyamines, combined gas chromatography-mass spectrometry has been employed. In Fig. 2-3 the mass spectra of caldine and thermine are compared to the correspondent analogues spermidine and spermine. Under electron impact all derivatives show weak but quite visible molecular ions (M^+) at m/e 419 and 433 for caldine and spermine respectively. The most intense fragment ions are obtained at m/e 126 for spermidine and m/e 154 for caldine, spermine and thermine. As might be expected from the close structural similarity of these compounds, their mass spectral fragmentation pattern are also closely related. The identity of caldine and thermine has been definitively established by comparing the mass spectra of these compounds with that of the corresponding reference compounds.

Quantitative estimation of polyamines.

In Table I are reported the amounts of polyamines present in *C. acidophila* cultures harvested during log-phase and stationary-phase of growth; the data have been obtained by isotope dilution technique. The absolute values of caldine and thermine, lacking the appropriate labeled carriers, were calculated assuming that their recoveries are similar to that of spermidine and spermine. Quantification of polyamines by gas-liquid chromatography gave results consistent with those reported in Table I.

DISCUSSION

The occurrence of 1,7 diamino-4-azaheptane in biological materials has

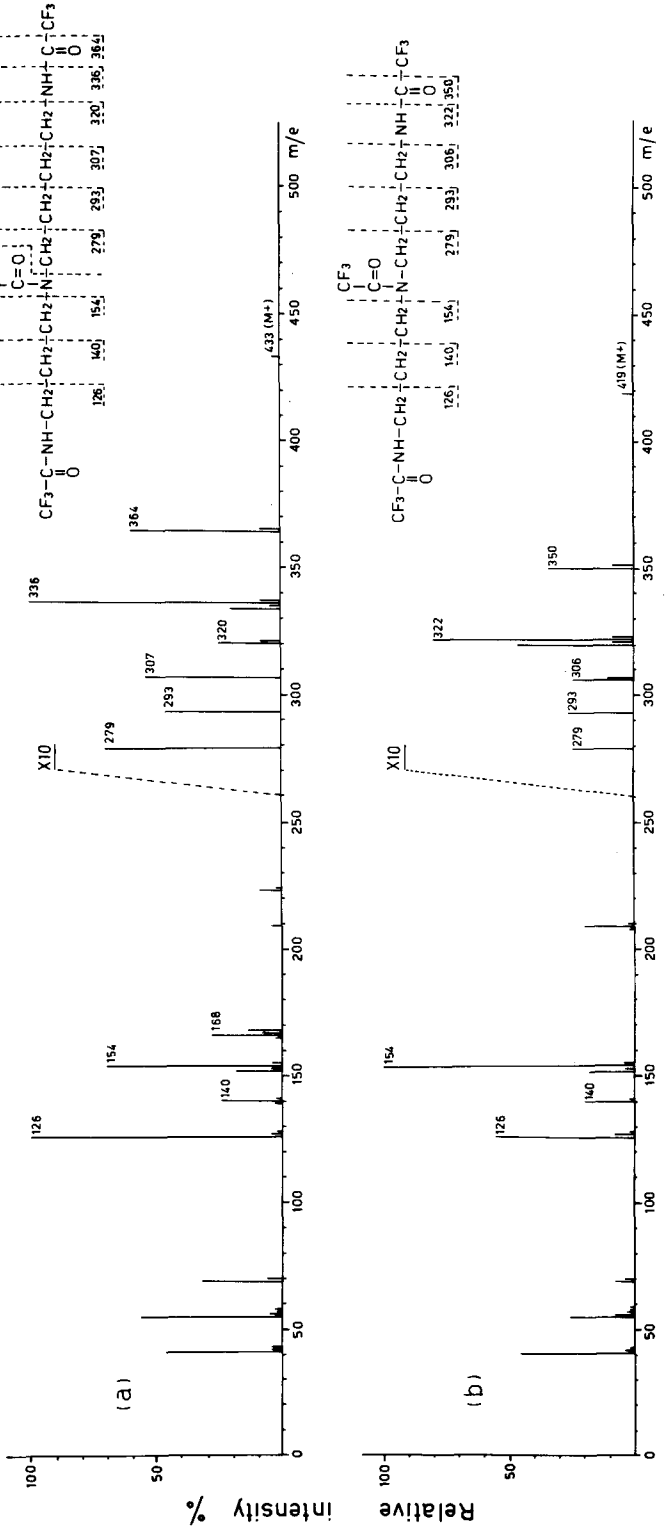


Fig.2. Normalized mass spectra of trifluoroacetylated spermidine (a) and caldine (b).

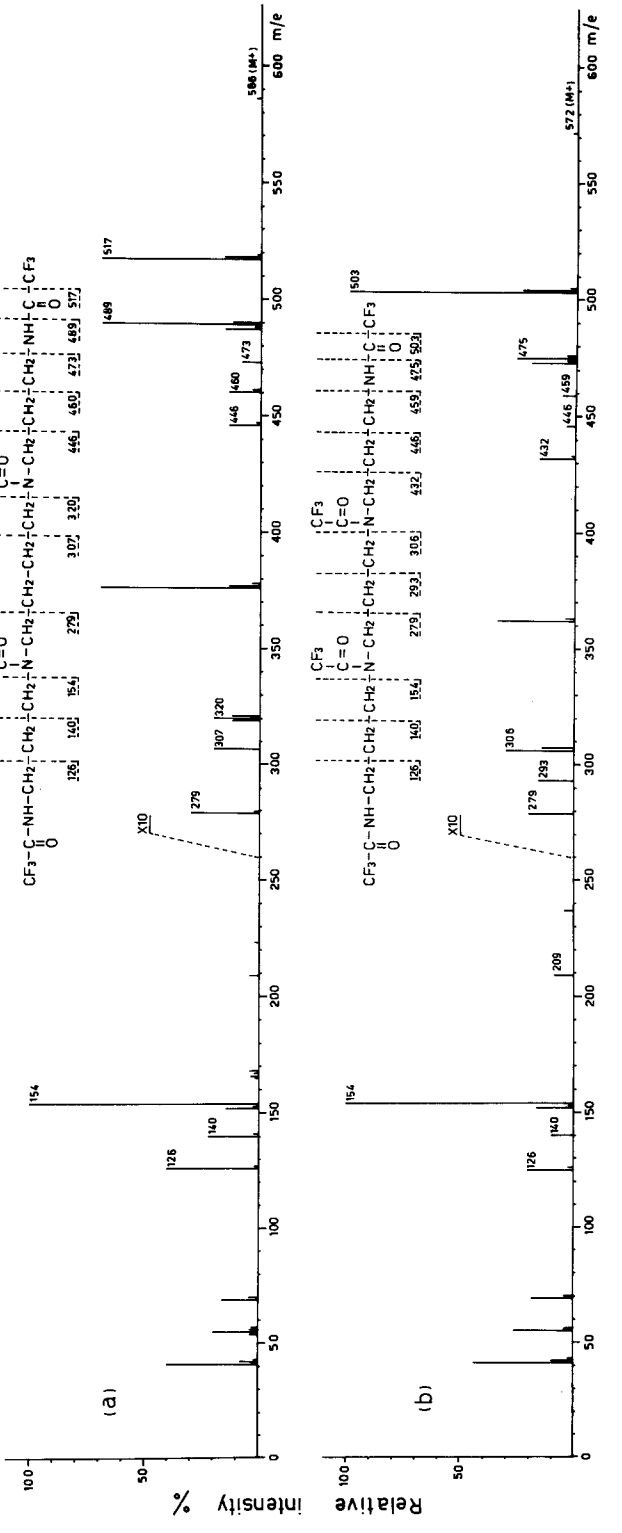


Fig.3. Normalized mass spectra of trifluoroacetylated spermine (a) and thermine (b).

TABLE I

Polyamine concentration in *Caldariella acidophila*

Polyamines	Log-phase	stationary-phase	Recovery[+]
	µmol/g wet cells		%
Putrescine	N.D.	N.D.	65
Spermidine	12.8	8.2	64
Caldine	2.0	1.8	/
Spermine	N.D.	N.D.	66
Thermine	8.1	15.6	/

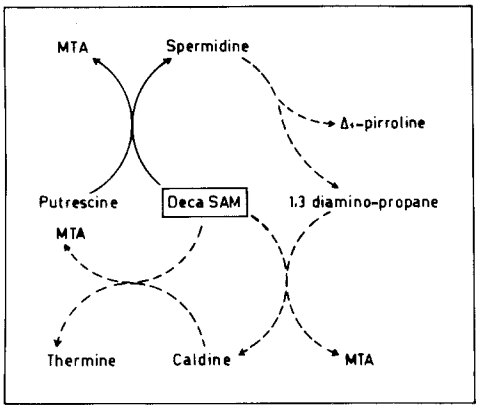
[+]Determined by isotope dilution technique (see Materials and Methods).
N.D.= not detectable.

been proved unequivocally for the first time in the present paper: the presence of this triamine in plant viruses was claimed in 1962 by Johnson and Markham (9), but more recent studies (10) could not confirm the result and questioned the biological role of the molecule.

The relatively high content of thermine (15.6 µmoles/g wet cells) in *C. acidophila* confirms with a different technique the recent report of Tairo Oshima on the presence of such molecule in extreme thermophiles (5) and adds more interest to the possible role(s) of this polyamine in relation to thermophily.

In our experimental conditions we were unable to detect any spermine in *C. acidophila*; this result, in apparent contrast with the elevated amounts of the polyamine in other thermophiles (1), could be related to the extremely high growth temperature of this microorganism.

The simultaneous presence of spermidine, caldine and thermine in the same microorganism is in agreement with the hypothesis that the triamine is presumably a biological precursor of thermine (5). As reported for several microorganisms spermidine could be oxidized into 1,3 diamino-propane and Δ_1-pirroline by a diamine oxidase; these compounds are generally considered to be the ulti-



Scheme 1. Proposed biosynthetic pathway of caldine and thermine in *Caldariella acidophila*.

mate oxidation products of polyamine catabolism. According to Scheme 1 1,3-diamino-propane could undergo a propylamine transfer reaction with S-adenosyl-5'-(3)methyltiopropylamine (DecaSAM) yielding methyltioadenosine (MTA) and caldine. This polyamine could in turn react with a second molecule of the sulfonium compound yielding MTA and thermine. The high concentration of DecaSAM recently reported in *C.acidophila* (11) is in accord with the hypothesis of propylamine transfer reactions between the sulfonium compound and 1,3 diamino-propane or caldine respectively. Studies to identify the correspondent propylamine transferase and its properties are in progress.

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